

Isolation and Characterization of Partial cDNA of Sucrose Synthase Putative Gene in Palmyra Palm (*Borassus flabellifer*)

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ABSTRACT

Intensification of biofuel resources is urgently needed considering decreased availability of world's fossil fuel. Palmyra palm (*Borassus flabellifer*) is highly potential to be developed as bioethanol source regarding the high sucrose content in its *nira*. It was observed that *nira* produced in dry season is sweeter than that in rainy season, which presumed to be influenced by a difference in expression level of sucrose-related genes during the two seasons. Study of Sucrose Synthase (SUS) gene of palmyra are therefore required prior to study of the gene expression. Palmyra SUS gene sequence is currently unavailable in GenBank, thereby pair of primers was designed from highly conserved region of SUS proteins among monocots. A 1866 bp partial cDNA fragment of SUS putative gene has been successfully isolated from RNA of the young leaves of *B. flabellifer*. BLASTn and BLASTp alignments showed that either *BfSUS* cDNA or *BfSUS* polypeptide has high similarity with SUS cDNA and proteins from diverse plant species with the highest similarity shown by *Tulipa gesneriana*. The phylogenetic tree showed that SUS protein sequences of monocot species were distinctively grouped and splitted from those of dicot species. The *BfSUS* was clustered in monocot group, although not specifically grouped with particular monocot species. Nevertheless, *B. flabellifer* showed nearest genetic distance with *Tulipa gesneriana* and *Oncidium cv. 'Goldiana'*. Characterization of *BfSUS* polypeptide using Geneious 4.6.2 indicated the presence of sucrose synthase (SUS) and glycosyl transferase (GT) domains, four putative UDP-glucose binding pockets within the GT domain, and a calcium-dependent Ser/Thr protein kinase binding site within the SUS domain. These domains and motifs are highly conserved in SUS proteins across plant species, confirming that the cDNA fragment obtained in this study is very likely cDNA encodes sucrose synthase in *B. flabellifer*.

Keywords: *Borassus flabellifer*, *nira*, sucrose, sucrose synthase, SUS domain, GT domain, Palmyra palm.

INTRODUCTION

Palmyra (*Borassus flabellifer*) is a *palmae* species with high potency to be developed as a source of bioethanol. The plant is typically grown in dry areas in strictly seasonal tropical or subtropical climate. The palmyra palm is known to be very adaptive in dry areas with only 500-900 mm annual rainfall (Flach and Rumawas, 1996). The main product of palmyra is the sweet liquid produced from its inflorescence, called *nira*, or locally known as *tuak* (Fox, 1977). The *nira* of palmyra palm contains 17-20% dry matters comprises of sucrose, amino acids, proteins, vitamins, and other essential minerals. Sucrose (C₆H₁₂O₆) that constitutes 13-18% per liter *nira* is the principal material in the production of bioethanol through the process of fermentation. It was observed in East Nusa Tenggara, particularly in Timor and Rote islands, that *nira* tapping is more

preferable in dry season rather than in rainy season. Beside the safety reason, it is probably because palmyra nira in dry season is sweeter than in rainy season. It becomes interesting to study whether this phenomena is solely caused by high level of water contained in the nira, by a higher rate of photosynthesis occurred in dry season, or it has something related with sucrose genes expression level. A preliminary study on sucrose related gene expressed in palmyra palm is therefore needed to answer the question.

Sucrose is the most important plant disaccharide; it is the principle form by which photosynthetic product is transported throughout plant tissues from the source photosynthetic tissue to the sink non-photosynthetic tissues (Bush, 1999). This disaccharide consists of one molecule of glucose and one molecule of fructose that is bounded by a glycosidic bond (Lodish *et al.*, 1999). Many enzymes involved in metabolism of sucrose. The closest related enzymes are sucrose-synthase (SUS: EC 2.4.1.13), sucrose phosphate synthase (SPS: EC 2.4.1.14), sucrose-6-phosphate-phosphatase (SPPase: EC 3.1.3.24), and invertase (EC 3.2.1.26). The reversible and irreversible reactions of sucrose hydrolysis are catalyzed, respectively, by SUS and invertase. The biosynthesis of sucrose is catalyzed by the sequential action of SPS and SPPase (Winter and Huber, 2000). Sucrose can be synthesized from hexose monophosphates by SUS or SPS. In the case of SUS, the *in vivo* sucrose concentrations are always much higher than fructose or UDP-glucose, resulting in reaction that is essentially always towards the direction of sucrose cleavage. Invertase, on the other hand, only catalyzes the cleavage of sucrose into glucose and fructose.

All enzymes catalyze transfer of sugar moieties from activated donor molecules to specific acceptor molecules by forming glycosidic bonds are classified in the glucosyltransferase (GT) family of enzyme. Sucrose synthase (EC 2.4.1.13) is a key enzyme involved in sucrose metabolism, included in the GT4 family regards to its role in transferring the glucose from UDP-glucose to fructose molecule to form sucrose or, in reverse, hydrolyze the sucrose into glucose and fructose. SUS enzyme plays significant role in food storage of many plants, either in the form of starch or sugar (Baud *et al.*, 2004). SUS was shown to control the mobilization of sucrose into various pathways that important for the metabolic, structural, and storage functions of the plant cell. Phloem-loading, a process by which sucrose is transported from photosynthetic to non-photosynthetic tissues, is facilitated by SUS. Inhibition of SUS-encoding genes had shown significant reduction in sucrose import capacity of floem tissues, thereby causing less sucrose content in tomato fruit (D'Aoust *et al.*, 1999). Conversion of sucrose to UDP-glucose that is catalyzed by SUS provides substrate for cell wall biosynthesis and starch synthesis in plant storage organs (Sun *et al.*, 1992; Zrenner *et al.*, 1995; Dejardin *et al.*, 1997; Hendrik, 1990). SUS activity also associated with development of nodules in legume plants and in regulation of apical meristem function (Craig *et al.*, 1999). Studies on SUS-encoding genes in many plant species revealed that *in vitro* environmental conditions, such as light, temperature, and water availability have a significant influence on the expression of SUS-encoding genes. Palmyra (*B. flabellifer*) which has higher sugar content in dry season than in wet season, probably reflects a likewise relation.

SUS genes have been isolated from various starch and sugar-storing plants, such as citrus (*CitSUS1* and *CitSUS2*) (Komatsu *et al.*, 2002), sugarcane (Kumar *et al.*, 2007), rice (Wang *et al.*, 1992), maize (*Sus1* and *Sh1* gene) (McCarty *et al.*, 1986), wheat (Marana *et al.*, 1988), cotton (Ruan *et al.*, 2003), tomato (Sun *et al.*, 1992), and potato (Zrenner *et al.*, 1995). The enzyme is found in all plant tissues but is highly expressed particularly in sink tissues (Baud *et al.*, 2004). According to those studies, the full length SUS gene has in average 4970 bp length, while its cDNA length is only about half of it, ranging from 2400 to 2500 bp. This research was aimed to study the characteristic of SUS gene in *B. flabellifer*. A comprehensive knowledge of the SUS gene characteristics is an initial requirement to study its expression level, and further to conduct manipulation or control of those genes, which enables efforts to enhance the productivity of *B. flabellifer* as a potential source of sugar and bioethanol.

RESULTS AND DISCUSSION

RNA Isolation from *Borassus flabellifer*

Total RNA of *B. flabellifer* was isolated from the young leaves of 35-40 years old-plants. Young leaves was chosen as it was reported that SUS genes are commonly expressed in newly developed tissues that require supply of either sucrose or sucrose derived-compounds (Kumar *et al.*, 2007). The results of five methods tested for RNA isolation from *B. flabellifer* is summarized in Table 1. The modified method of Apt *et al.* (1995) resulted in good quality of RNA although the purity and quantity were rather low. The RNA isolation was best accomplished by using TRIzol[®] Reagent (Invitrogen). The reagent, a mono-phasic solution composed of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). This method successfully generated two distinct bands of 18s and 28s ribosomal RNA that were clearly occurred in 1% gel electrophoresis (Figure 1), which indicated a good RNA quality. The RNA yield was relatively high, 1600 µg/ml, with low protein contamination as shown by the ratio 1.62. We noted that the success in obtaining high RNA yield from *Borassus* leaves is greatly determined by the finest powder that could be recovered from the leaves tissue.

Reverse Transcription- PCR

A total cDNA from the young leaves of *B. flabellifer* were obtained by cDNA synthesis. They then used as template for the next PCR reaction. The annealing of SUS-specific primers to the target cDNA were best achieved in temperature 52°C. As well, 2 mM of Mg²⁺ and 1.5 µg/50 µl of template cDNA were found to be the most suitable concentration for the PCR mix. Total 30 cycles of amplification using the gene specific primers, SSFw and SSRv, has successfully produced a single fragment of 1866 bp that was detected in 1% agarose gel (Figure 2). According to the expected product size, this 1866 bp fragment was predicted as the target fragment of the SUS gene.

Cloning of SUS Putative Gene and Transformation of *E. coli*

Prior to sequencing reaction, the SUS putative fragment is purified from the agarose gel and then cloned into a plasmid vector, pGEM[®]-T easy (PROMEGA), which then delivered to *E. coli* strain DH5α. *E. coli* cells that had been transformed were able to grow in the ampicillin-containing medium. Transformed cells containing SUS gene fragment were selected by blue-white screening method. Transformation of *E. coli* strain DH5α with pGEM[®]-T containing SUS fragment resulted in blue and white colonies growth on the ampicillin contained-LB media.

Plasmid Isolation and Confirmation of Gene Insertion

Plasmid isolation from the *E. coli* white colony and subsequent restriction cut using particular restriction enzymes are purposed to confirm the presence of the SUS gene fragment within the plasmid of *E. coli*. When cut with *EcoRI*, the plasmid produced two unexpected fragments, ± 1300 bp and ± 500 bp, instead of 1866 bp (Figure 3a). It was presumed that the SUS fragment of *B. flabellifer* has an *EcoRI* restriction site within its sequence, thereby produced shorter fragment than expected. It was found later that the sequence of SUS gene of *B. flabellifer* does have the recognition site for *EcoRI*. The plasmid was then cut with another endonuclease enzyme, *NotI*. As expected, cutting with *NotI* generated a single 1866 bp fragment (Figure 3b). This result indicated that the SUS gene fragment had been successfully inserted into the pGEM[®]T easy plasmid.

PCR screening with SP6 and T7 primers was further conducted to prove the presence of SUS fragment within the multiple cloning sequence (MCS) region in the plasmid. Those two primers

flank the MCS region in the pGEM[®]-T easy plasmid. As expected, a ± 1995 bp fragment was occurred (Figure 3c). This length corresponds to the length of the SUS fragment, ± 1866 bp, plus 129 bp lengths of the SP6 and T7 MCS region.

Sequencing and Characterization of SUS Putative Gene and Polypeptide

Two steps of sequencing were required since SUS putative fragment of *B. flabellifer*, further stated as *BfSUS*, is relatively long for a single reading by the sequencer system. The SP6 and T7 primers were used in the former sequencing, followed by specific internal primers to read the gap sequence within the fragment. The later primers were designed from the read of the former sequence. Using Geneious 4.6.2 program, an overlapped reading occurred from those two steps of sequencing, thus finally assembly the whole sequence of the *BfSUS* putative gene fragment. The assembled *BfSUS* fragment was trimmed from pGEM[®]T vector region by using VecScreen program (www.ncbi.nlm.nih.gov), followed by determination of SSFw and SSRv primers annealing region. The final construction of *BfSUS* partial gene, which total length is 1866 bp, was successfully obtained (Figure 4).

The sequence of *BfSUS* putative gene was first analyzed by BLASTn program (Altschul *et al.*, 1997) to figure out the similarity level of *BfSUS* putative gene with other *SUS* gene sequences recorded in the GenBank. The percentage of identical sites and query coverage indicated that *BfSUS* putative gene shares quite high similarity, 70.8% – 80.6%, with *SUS* genes from various monocots. Thus, confirming that the isolated 1866 bp *BfSUS* fragment is most likely a sucrose synthase gene. *BfSUS* sequence shows the highest similarity with *SUS* genes of *Tulipa gesneriana* (80.6%), followed by *X. mokara* (78.8%), *Potamogeton distinctus* (78.7%), *Oncidium sp. cv Goldiana* (78.6%), and *Bambusa oldhamii* (78.5%).

Translation of *BfSUS* cDNA sequence resulted in BfSUS polypeptide sequence consists of 622 amino acids (Figure 5). The BLASTp program showed that *BfSUS* polypeptide sequence is highly similar with SUS protein sequences of many plant species, ranged from 78.3 % to 87.5%, confirming that the BfSUS gene expresses SUS protein. The highest similarity, 87.5%, are showed by *Tulipa gesneriana*, a *Liliaceae* plant widely known as *tulip*, and *Oncidium cv 'Goldiana'*, a genus that contains about 330 species of orchids from the *Orchidaceae* family. *Oryza sativa* (86.8%), *Bambusa oldhamii* (86.5%), *Zea mays* (86.2%), and other monocots also showed high similarity.

In order to elucidate relationship between BfSUS putative protein and SUS proteins from other species, and between monocot and dicot species, a phylogenetic tree is generated using Neighbor-Joining method (Figure 6). The following things can be interpreted from the phylogenetic tree. First, the similarity of SUS polypeptide sequence in certain taxonomic group tends to be higher than another group. The similarity of SUS among monocot species, for instance, is distinctively higher than those of dicot species. This also showed by species coming from the same genus, for instance, between *Solanum tuberosum* and *Solanum lycopersicum*. Second, *B. flabellifer* is shown to be clustered with the monocot group, although not specifically grouped with particular monocot species. It is however shown that *Borassus flabellifer* has the nearest genetic distance with *Tulipa gesneriana*. Although these two species live in distinctively different climate regions, presumably they both evolve similar mechanism to adapt to osmotic stress in their surroundings, probably by accumulating sucrose. In water stress conditions, plants are able to keep their osmotic gradient lower than their surrounding by accumulating more solutes, including sucrose, in their tissues, thereby preventing loss of water.

Multiple sequence alignment of SUS polypeptides indicated two main domains that typically occurred in all plant species. Those are a *sucrose synthase* domain located upstream toward the N-terminal and a *glucosyltransferase* domain that is located downstream towards the C-terminal

of the protein. These domains differs SUS from other glycosyltransferase enzymes. Using *pFam* database, it was shown that those two domains are occurred in the *BfSUS* polypeptide albeit in partial length. The moiety of sucrose synthase and glucosyltransferase domain is found toward the N and C-terminal region, respectively, of the *BfSUS* polypeptide. The overlapped region between these domains is spanned from Asn283 to Ser559. Despite found to be highly conserved among plant species, the structural and functional sites and motifs within sucrose synthase domain are still unrevealed yet. Instead, four putative functional motifs have been reported for the second domain, the glycosyl transferase.

It is known that structure of a protein determine its function. Therefore, function of a protein could be deduced by comparing protein sequences and structures with homolog proteins of known function. Similar motifs between two proteins generally will have same function, especially when they are homolog (Horton *et al.*, 2006). Functional motifs within the *BfSUS* polypeptide were elucidated by finding functional conserved motifs within other enzymes of GT family which had been previously annotated. Buschiazzo *et al.* (2004) had successfully isolated and crystallized another GT family enzyme from *Agrobacterium tumefaciens*, the Glycogen Synthase (GS). GS catalyses the synthesis of the α -1,4-glucose backbone in the reaction of glycogen biosynthesis. This enzyme possesses the GT domain, a domain also found in SUS enzyme, with some annotated motifs within it. According to homolog motifs in GS (Buschiazzo *et al.*, 2004), four functional motifs predicted as urasile diphosphate (UDP)-binding pockets were detected in the *BfSUS* polypeptide.

The first putative UDP binding pocket found in *BfSUS* polypeptide is Gly169, a residue located in a glycine rich motif, 166-DTGGQ-170 (Figure 7) (Huber and Huber, 1996; Buschiazzo *et al.*, 2004). The Glycine (G) residue is predicted to directly contact with phosphate group of UDP molecule that binds to SUS protein. Buschiazzo *et al.* (2004) discovered that, in the close conformation of the GS protein, the glycine residues in the KXGGL motif come into contact with the phosphate groups of UDP. This role had been reported also in the GS of *Escherichia coli* (Furukawa *et al.*, 1993). They reported that only the glycine residues but not the basic side chain appear to be essential for GS enzymatic activity. The DTGGQ motif was found to be conserved in sucrose phosphate synthase (SPS), another member of GT family, which functioned as fructose-6-P binding site (Huber and Huber, 1996).

The three subsequent UDP-putative binding pockets in *BfSUS* polypeptide are 444-MAR-446 residues, N520, and T546 (Figure 8 and 9). It was observed that the MAR motif within the *BfSUS* polypeptide is located at the C-terminal end of a β -strand (Figure 8), a pattern that is also occurred in ISR motif of GS polypeptide, albeit in different number of β -strand. This probably reflects their similar role. It was observed that the guanidinium group of R299, in GS, interacts with the phosphate group of ADP via a hydrogen bonding (Buschiazzo *et al.*, 2004). The MAR residues of *BfSUS* polypeptide thereby suggested as binding site for phosphate group of UDP via a hydrogen interaction. The Asn520 (N520) motif of *BfSUS* polypeptide is predicted to bind with the adenine ring of the UDP molecule. According to GS, the carbonyl group of protein backbone in Asn520 may be interacted with atom N6 of the adenine ring of UDP molecule via a weak hydrogen interaction. The last putative UDP binding pocket, the T546 was suggested to bind ribose sugar of UDP molecule. The side chain of T546 may interact via hydrogen bond with O₂ atom of the ribose sugar.

The last conserved region of SUS protein that is also found in *BfSUS* polypeptide is the Ser170 putative phosphorylation site. This essential site was found within a typical serine residue containing- motif, 'RHLSS', which lay between Arg167 and Ser171. In the partial *BfSUS* polypeptide this motif is located between Arg30 and Ser34 (30-RHLSS-34). This motif was firstly detected in *Zea mays* SUS protein, spanned from Arg159 to Ser163 (Hardin *et al.*, 2003). This motif was also found in *Oryza sativa*, *Saccharum officinarum* (R159 to S163), *Bambusa*

oldhamii, *Triticum aestivum* (R167 to S171), and *Tulipa gesneriana* (R161 to S165). Hardin *et al* (2003) reported that SUS protein of *Zea mays* is phosphorylated by calcium-dependent protein kinases (CDPKs) at the Ser170 residue, within the RHLSS motif, in addition to Ser15 (Asano *et al.*, 2002). Ser15 was reported as a major phosphorylation site that affects cleavage activity and membrane association, whereas Ser170 is a minor phosphorylation site that may trigger enzyme degradation via the ubiquitin/26S proteasome (Qiu *et al.*, 2007). Since phosphorylation of Ser170 is important for enzyme degradation but not directly involved in the catalytic activity of SUS, this site is suggested as one of the allosteric sites of the enzyme.

Those all results confirm that the isolated 1866 bp of *BfSUS* gene fragment is most likely the sucrose synthase- encoding gene in *B. flabellifer* that encodes SUS protein. Further expression study however is required to prove that the *BfSUS* gene actually expresses sucrose synthase protein in *B. flabellifer*. The sequence of partial *BfSUS* has been submitted to the GenBank with accession number **GQ265926**.

MATERIALS AND METHODS

Plant Material

Young leaves were picked from the apical shoot of 35-40 years age-plant of Palmyra palm (*Borassus flabellifer*) grown in Lasiana shore area in Kupang, East Nusa Tenggara. Leaves samples were cleaned, wrapped, and immediately stored in cold condition before being taken to Bandung. The whole processes of sampling were done with RNase-free standard work procedure. Leaves samples were then frozen in liquid nitrogen and were stored in -80°C refrigerator before RNA isolation. All the laboratory works took place in the Laboratory of Genetics and Molecular Biology, in SITH-ITB, Bandung.

RNA Isolation

Total RNA of *B. flabellifer* was extracted from the fresh young leaves that were previously stored in -80°C. All chemicals were diluted in RNase-free water and prepared using RNase-free equipments. All equipments were formerly treated with diethylpyrocarbonate (DEPC) before use. Grinding equipment, such as mortar, pestel, and scissors were chilled before use, either by storing in -80°C or by soaking in liquid nitrogen.

Five protocols have been tested to optimize RNA isolation method from young leaves of *B. flabellifer*. Those are method of Seki *et al.* (2002), method of Khemvong and Suvachittanont (2005), modified-method of Apt *et al.* (1995), TRIzol-modified (I) method, and TRIzol modified (II) method (Invitrogen). Quality of RNA is determined by absorbance ratio in 260 nm and 280 nm UV light in the range of 1.80 – 2.00 which indicated low contamination of protein. Appearance of two typical ribosomal RNA bands, 18s and 28s, in electrophoresis gel also indicated good quality of RNA. The quantity of RNA was determined by RNA yield (µg) per total solution volume (ml).

Primer Design

Sequence of *B. flabellifer* SUS gene is currently unavailable in the GenBank, thereby pair of primer was designed from highly conserved region of SUS proteins among monocot plant species. Sucrose synthase protein sequences of *Oryza sativa*, *Zea mays*, *Saccharum officinarum*, and other monocots, were accessed from NCBI GenBank database (www.ncbi.nlm.nih.gov). Multiple sequence alignment of SUS proteins was conducted using Clustal X software. The most conserved regions in SUS proteins among those species were chosen for primer sequence

determination. Primer sequences were deduced from those regions by choosing a particular group of species whose cDNA sequence is highly conserved. This was accomplished by BioEdit and CodeHop programs. Nucleotide number 412 to 432 of *SUS* gene of *Zea mays* was chosen as forward primer; while reverse primer was taken from nucleotide number 2277 to 2252. Primer physical characteristics were measured by *sigma-genosys* primer calculator. Specificity of both primer pairs were tested by nucleotide BLAST program (Altschul *et al.*, 1997). The chosen primer sequences showed eligible characteristics, and more importantly, exhibited high specificity for *SUS* gene (Table 2).

Reverse Transcription-PCR

Isolation of sucrose synthase (*SUS*) gene from *B. flabellifer* was accomplished by *two step* reverse-transcription polymerase chain reaction (RT-PCR) method. In the first step, total cDNA is synthesized from the total RNA by using *Superscript II cDNA synthesis kit* (Invitrogen). In the second step, PCR amplification using *SUS* gene-specific primers was carried out to specifically amplify fragment of *SUS* gene from the previously isolated cDNA.

Total cDNA synthesis

Total cDNA synthesis of *B. flabellifer* was conducted using *Superscript II cDNA synthesis kit* (Invitrogen). Composition of each component in cDNA synthesis reaction is described in Table 3. All solutions are diluted in DEPC-treated deionized water. All steps were carried out according to RNase-free standard work procedure to avoid or minimize degradation of the RNA.

A master mix solution was prepared by mixing buffer RT with $MgCl_2$, Dithiothreitol (DTT), and RNase outTM recombinant RNase. Separately, RNA sample was mixed with oligo dT primer and dNTPs prior to five minutes incubation in 65°C and then one minute in ice. The master mix was then added to the RNA sample mixture and then diluted with DEPC-treated water to 20 µl final volume. This final mixture was incubated for two minutes in 42°C for primer annealing. SuperscriptTM II- Reverse Transcriptase was subsequently added to the mixture, gently homogenized, and then incubated for 50 minutes in 42°C for complete reaction of cDNA synthesis. The reaction was terminated by 15 minutes incubation in 70°C. To eliminate any remaining RNA strands in the cDNA solution, RNaseH were added to the solution and then incubated in 37°C for 20 minutes. Total cDNA of *B. flabellifer* young leaves was obtained at the end of this step.

PCR Amplification for Isolation of *SUS* gene

A standard PCR amplification technique was conducted following the cDNA synthesis to specifically isolate the *SUS* gene. Optimization of PCR profile, especially annealing temperature, was carried out to find the best condition that favor specific amplification of the *SUS* gene fragment. A range of annealing temperature, from 45°C to 55°C, was tested. Concentration of cDNA template, ranging from 0.5 to 2 µg /50 µl, and Mg^{2+} , ranging from 1 to 2.5 mM, were tested as well to obtain best fragment of the target gene. The PCR reaction was carried out using a *Long PCR Enzyme Mix* product (FERMENTAS). Composition of the PCR mixture is described in Table 4.

Gene Cloning and Transformation of *E. coli*

The product of RT-PCR was observed by electrophoresis using 1% agarose gel. Detected *SUS* fragment was purified from the agarose gel and then cloned to the pGEM-T Easy® vector by employing T4 ligase enzyme. To amplify the *SUS* fragment, the recombinant plasmids were subsequently delivered into bacterial cell of *Escherichia coli* strain DH5α using heat-shock

treatment. The transformed cells of *E. coli* that contain recombinant plasmid were selected through screening of blue-white colony. The transformed cells were cultured for 16 hours in Luria-Bertany (LB) media containing 0.1 ng/μl ampicillin. The plasmids DNA of the transformed cells were then extracted by alkaline lysis method (Sambrook *et al.*, 1989). Insertion of SUS fragment into plasmid was confirmed by cutting the plasmid with two endonuclease restriction enzymes, *EcoRI* and *NotI*.

Sequencing and Characterization of BfSUS Gene and Polypeptide

SUS putative fragment that have been cloned to pGEM-T easy plasmid were further analyzed by dideoxy chain-termination method to read the nucleotide sequence of the isolated fragment. Minimum 150 ng/μl of plasmid DNA is required to a single sequencing reaction. Since the 1866 bp gene fragment is relatively long for a single accurate reading by the sequencer system, two steps of sequencing are needed to accomplish the total fragment reading. The first sequencing employed T7 and SP6 universal primers, which followed by second sequencing using gene internal-primers. Gene-internal primers were designed from the first sequencing result. Sequencing result from those four primer directions were finally assembled using Geneious 4.6.2 program. All sequencing process was conducted by MacroGen Inc. in South Korea.

Characterization of *BfSUS* putative gene comprises nucleotide and polypeptide sequence analysis. The nucleotide sequence of the putative *BfSUS* was aligned with SUS cDNA sequences deposited in GenBank (www.ncbi.nlm.nih.gov) using BLASTn program in order to find their similarity. The *BfSUS* cDNA sequence was then translated into BfSUS polypeptide using Geneious 4.6.2, followed by searching in the GenBank using BLASTp program. The highly conserved regions, including essential domains and motifs, were searched within BfSUS putative polypeptide, either by employing Geneious 4.6.2 program or by comparing the polypeptide with other related proteins that have been previously annotated. Other proteins from the same family are preferable since it is expected to share similar properties with the SUS. To reveal genetic relationship between *Borassus flabellifer* and other plant species based on their SUS polypeptide sequence, a neighbor-joining (NJ) phylogenetic tree was generated using Geneious 4.6.2 tree builder.

CONCLUSION

A partial cDNA fragment of sucrose synthase (SUS) putative gene has been successfully isolated from the young leaves of *B. flabellifer*, henceforth named as *BfSUS*. The sequence has been submitted to the Genbank with accession number **GQ265926**. The sequence of *BfSUS* cDNA, and the corresponding BfSUS polypeptide, is highly similar with *SUS* cDNA and *SUS* protein from various plant species recorded in GenBank, with highest similarity shown by *Tulipa gesneriana* and *Oncidium sp.* The *BfSUS* putative polypeptide performs all typical characteristics of *SUS* protein, including most conserved domains and motifs. Presence of sucrose synthase (*SUS*) and glycosyl transferase (*GT*) domains, four putative UDP-glucose binding pockets within the *GT* domain, and a calcium-dependent Ser/Thr protein kinase binding site (Ser170) within the *SUS* domain, confirms that the *BfSUS* cDNA fragment obtained in this study is most likely encodes sucrose synthase in *B. flabellifer*. This also assuring that *BfSUS* gene is expressed in the young leaves of *B. flabellifer*.

Table 1. Summary of five tested methods for RNA isolation from young leaves of *B. flabellifer*. TRIzol- modified II found to be the best method for RNA isolation from the young leaves of *B. flabellifer*

M e t h o d	Ratio of UV absorbance (A_{260}/A_{280})	RNA quality in gel electrophoresis	RNA yield ($\mu\text{g/ml}$) ^{*)}	Total time (hour)
TRIzol-modified I (Invitrogen)	1.23 – 1.24	Average	114.75	5
Seki <i>et al.</i> , 2002	1.44 – 1.47	Poor	93.50	8
Khemvong &Suvachittanont (2005)	1.67 – 2.00	Poor	44.625	12
Apt <i>et al.</i> (1995)-modified	1.45 – 1.47	Good	148.75	18
TRIzol-modified II (Invitrogen)	1.61 – 1.62	Good	1600	4

^{*)} RNA yield = (Absorbance in 260 nm) x (42.5 $\mu\text{g/ml}$) x (dilution factor)

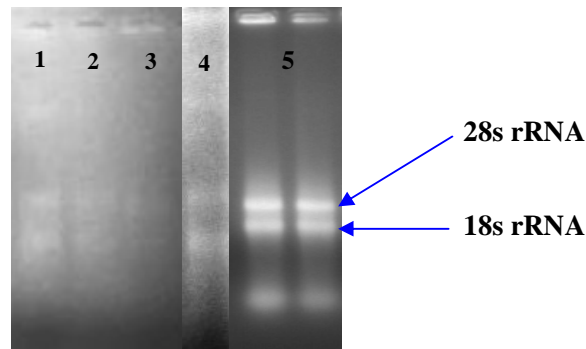


Figure 1. Comparison of RNA quality obtained by the five tested methods. Two bands of ribosomal RNA, the 18s and 28s rRNA, that were occurred in electrophoresis gel indicated that total RNA of *B. flabellifer* has been successfully obtained. TRIzol-modified II method is the best method for RNA isolation from the young leaves of *B. flabellifer*.

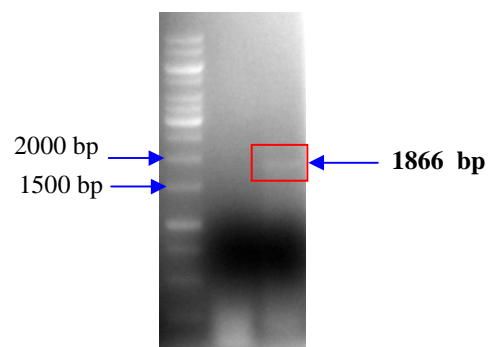


Figure 2. A single cDNA fragment of 1866 bp, generated by reverse transcription-PCR, predicted as the target fragment of the SUS gene of *B. flabellifer*.

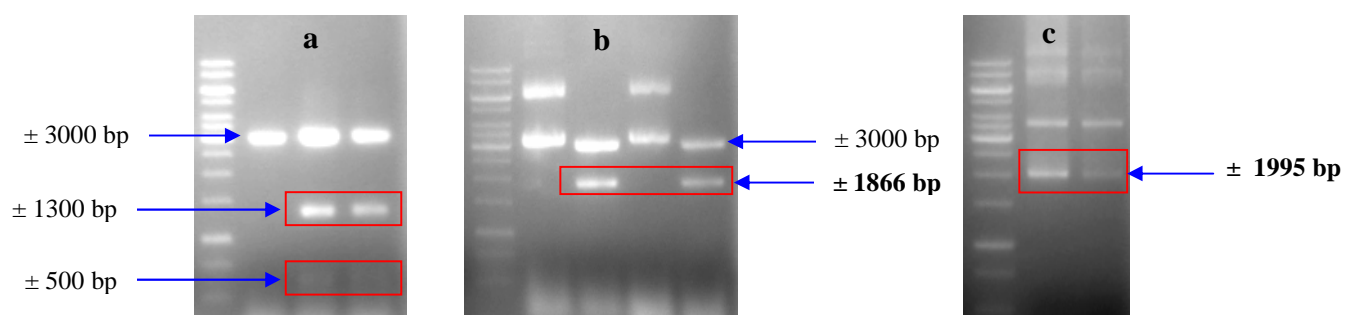


Figure 3. (a) The recombinant plasmid cut with *EcoRI* generated two unexpected ± 1300 and ± 500 bp fragments indicating gene internal cut by this enzyme; (b) plasmid cut with *NotI* generated a single expected fragment of length ± 1866 bp, indicating that SUS gene of *B. flabellifer* had been successfully inserted in the MCS region of the plasmid; (c) PCR screening using SP6 and T7 primers resulted in ± 1995 bp fragment confirmed the presence of SUS fragment within the MCS region of the pGEM[®]-T easy plasmid.

Table 2. Pair of forward and reverse primers used to isolate sucrose synthase gene from total RNA of *Borassus flabellifer* young leaves.

Primer Code	Primer Sequence	Start	Primer Length	Product Size (bp)	GC (%)	Tm (°C)	Sec. Structure	BLAST Output (Σ species)
SUSFw	CTTGAGCTGGACTTTGAGCCA	412	21	1866	52.38	62.92	Weak	10
SUSRv	CTTCCAGGTGTACTTCTCCTCGATAC	2277	26		50.00	62.72	Weak	8

Table 3. Chemical composition in cDNA synthesis

No.	Component	Final concentration
1	Total RNA of <i>B. flabellifer</i>	2 μ g/20 μ l
2	dNTPs	0.5 Mm
3	Oligo (dT) ₁₂₋₁₈ primer	0.025 μ g/ μ l
4	Buffer RT	1x
5	MgCl ₂	5 mM
6	Dithiothreitol (DTT)	0.01 M
7	RNase out TM Recombinant RNase	1 u/20 μ l
8	Superscript TM II- Reverse Transcriptase	1u/20 μ l
9	DEPC-treated deionized water	until 20 μ l
10	<i>E. coli</i> RNaseH	1 μ l/20 μ l

Table 4. Composition of PCR mixture for SUS cDNA isolation

No.	Component	Final concentration
1	10x long PCR buffer	1x
2	2 mM dNTP mix	0.2 mM
3	25 mM MgCl ₂	2 mM
4	Primer (forward and reverse)	0.2 μ M
5	Total cDNA	0.03 μ g/ μ l
6	Long PCR enzyme mix	1.25 unit/ 50 μ l
7	Nuclease free water	to 25 μ l

1	CTT	GAG	CTG	GAC	TTT	GAG	CCA	TTC	AAT	GCT	TCC	TTT	CCT	CGG	CCT	45
1	Leu	Glu	Leu	Asp	Phe	Glu	Pro	Phe	Asn	Ala	Ser	Phe	Pro	Arg	Pro	15
46	TCA	CTG	TCG	AAA	TCC	ATT	GGT	AAT	GGA	GTG	CAG	TTC	CTC	AAT	CGC	90
16	Ser	Leu	Ser	Lys	Ser	Ile	Gly	Asn	Gly	Val	Gln	Phe	Leu	Asn	Arg	30
91	CAC	CTC	TCT	TCA	AAA	CTG	TTT	CAT	GAC	AAA	GAA	AGC	ATG	TAC	CCA	135
31	His	Leu	Ser	Ser	Lys	Leu	Phe	His	Asp	Lys	Glu	Ser	Met	Tyr	Pro	45
136	CTG	CTT	AAT	TTT	CTT	CGG	GCA	CAC	AAA	TAT	AAG	GGA	ATG	ACA	ATG	180
46	Leu	Leu	Asn	Phe	Leu	Arg	Ala	His	Lys	Tyr	Lys	Gly	Met	Thr	Met	60
181	ATG	TTA	AAT	GAT	AGA	ATA	CAA	AGC	CTG	AGC	GCT	CTC	CAA	GCT	GCA	225
61	Met	Leu	Asn	Asp	Arg	Ile	Gln	Ser	Leu	Ser	Ala	Leu	Gln	Ala	Ala	75
226	TTA	AGA	AAG	GCA	GAG	GAA	TAT	CTG	TTG	AGT	ATC	CCA	GCA	GAC	ACT	270
76	Leu	Arg	Lys	Ala	Glu	Glu	Tyr	Leu	Leu	Ser	Ile	Pro	Ala	Asp	Thr	90
271	CCT	TAT	TCG	GAG	TTT	AAT	CAC	AGG	TTT	CAA	GAG	CTT	GGT	TTA	GAG	315
91	Pro	Tyr	Ser	Glu	Phe	Asn	His	Arg	Phe	Gln	Glu	Leu	Gly	Leu	Glu	105
316	AAG	GGT	TGG	GGT	GAT	ACA	GCT	CAG	CGT	GTT	GGT	GAG	ACT	ATT	CAT	360
106	Lys	Gly	Trp	Gly	Asp	Thr	Ala	Gln	Arg	Val	Gly	Glu	Thr	Ile	His	120
361	CTA	CTC	CGT	GAT	CTT	CTT	GAG	GCA	CCT	GAT	CCT	TGC	ACC	CTA	GAG	405
121	Leu	Leu	Arg	Asp	Leu	Leu	Glu	Ala	Pro	Asp	Pro	Cys	Thr	Leu	Glu	135
406	AAA	TTT	CTT	GGG	ACA	ATT	CCT	ATG	GTC	TTT	AAT	GTT	GTC	ATT	TTA	450
136	Lys	Phe	Leu	Gly	Thr	Ile	Pro	Met	Val	Phe	Asn	Val	Val	Ile	Leu	150
451	TCT	CCA	CAC	GGT	TAC	TTT	GCC	CAA	GCT	AAT	GTT	TTG	GGA	TAC	CCT	495
151	Ser	Pro	His	Gly	Tyr	Phe	Ala	Gln	Ala	Asn	Val	Leu	Gly	Tyr	Pro	165
496	GAC	ACT	GGA	GGC	CAG	ATT	GTC	TAT	ATT	TTG	GAT	CAA	GTT	CGT	GCA	540
166	Asp	Thr	Gly	Gly	Gln	Ile	Val	Tyr	Ile	Leu	Asp	Gln	Val	Arg	Ala	180
541	CTA	GAG	AGT	GAG	ATG	CTT	CTT	AGA	ATG	AAG	CAG	CAA	GGT	CTC	AAT	585
181	Leu	Glu	Ser	Glu	Met	Leu	Leu	Arg	Met	Lys	Gln	Gln	Gly	Leu	Asn	195
586	ATC	ACT	CCT	CGA	ATT	CTT	ATT	GTA	ACT	AGA	TTA	CTA	CCT	GAT	GCA	630
196	Ile	Thr	Pro	Arg	Ile	Leu	Ile	Val	Thr	Arg	Leu	Leu	Pro	Asp	Ala	210
631	ATT	GGG	ACC	ACT	TGC	GGT	CAG	CGG	CTT	GAG	AAA	GTC	CTA	GGC	ACA	675
211	Ile	Gly	Thr	Thr	Cys	Gly	Gln	Arg	Leu	Glu	Lys	Val	Leu	Gly	Thr	225
676	AAG	CAC	ACG	CAC	ATT	CTG	CGG	GTT	CCA	TTT	AGA	AAT	GAA	AAG	GGA	720
226	Lys	His	Thr	His	Ile	Leu	Arg	Val	Pro	Phe	Arg	Asn	Glu	Lys	Gly	240
721	ATC	CTT	CGC	AAA	TGG	ATC	TCA	CGC	TCC	GAT	GTA	TGG	CCT	TAC	CTT	765
241	Ile	Leu	Arg	Lys	Trp	Ile	Ser	Arg	Ser	Asp	Val	Trp	Pro	Tyr	Leu	255
766	GAA	ACT	TAT	GCA	GAG	GAT	GTT	GCA	AAT	GAA	CTG	GCT	GGA	GAA	CTG	810
256	Glu	Thr	Tyr	Ala	Glu	Asp	Val	Ala	Asn	Glu	Leu	Ala	Gly	Glu	Leu	270
811	CAG	GCA	ACC	CCG	GAT	CTC	GTT	ATT	GGA	AAC	TAC	AGT	GAT	GGA	AAC	855
271	Gln	Ala	Thr	Pro	Asp	Leu	Val	Ile	Gly	Asn	Tyr	Ser	Asp	Gly	Asn	285
856	CTA	GTA	GCA	TCT	TTG	TTG	GCA	CAT	AAA	CCA	GGG	GTT	ACT	CAG	TGT	900
286	Leu	Val	Ala	Ser	Leu	Leu	Ala	His	Lys	Pro	Gly	Val	Thr	Gln	Cys	300
901	ACT	ATT	GCC	CAT	GCC	TTG	GAG	AAA	ACA	AAG	TAT	CCA	AAT	TCA	GAT	945
301	Thr	Ile	Ala	His	Ala	Leu	Glu	Lys	Thr	Lys	Tyr	Pro	Asn	Ser	Asp	315

946	ATA TAC TGG AAA AAG TTT GAG AAC CAG TAC CAT TTT TCT TCT CAG	990
316	Ile Tyr Trp Lys Lys Phe Glu Asn Gln Tyr His Phe Ser Ser Gln	330
991	TTT ACT GCT GAT TTA ATT GCT ATG AAT CAT GCT GAT TTC ATT ATC	1035
331	Phe Thr Ala Asp Leu Ile Ala Met Asn His Ala Asp Phe Ile Ile	345
1036	ACC AGT ACT TTC CAA GAG ATT GCC GGA AGC AAG GAC ACC GTT GGG	1080
346	Thr Ser Thr Phe Gln Glu Ile Ala Gly Ser Lys Asp Thr Val Gly	360
1081	CAG TAC GAG TCT CAC ATT GCA TTT ACC CTC CCT GGG CTC TAC CGA	1125
361	Gln Tyr Glu Ser His Ile Ala Phe Thr Leu Pro Gly Leu Tyr Arg	375
1126	GTG GTT CAT GGA ATT GAT GTA TTT GAC CCA AAG TTC AAC ATT GTT	1170
376	Val Val His Gly Ile Asp Val Phe Asp Pro Lys Phe Asn Ile Val	390
1171	TCC CCT GGA GCT GAT ATG TCT ATT TAC TTC CCT TAC ACG GAA GAG	1215
391	Ser Pro Gly Ala Asp Met Ser Ile Tyr Phe Pro Tyr Thr Glu Glu	405
1216	AGT AAG AGA TTG ACT TCT CTT CAC CCT GAA ATT GAG GAG CTC CCC	1260
406	Ser Lys Arg Leu Thr Ser Leu His Pro Glu Ile Glu Glu Leu Pro	420
1261	TTC AGT TCT GTG GAG AAC TCT GAA CAC AAG TTT GTA TTG AAG GAC	1305
421	Phe Ser Ser Val Glu Asn Ser Glu His Lys Phe Val Leu Lys Asp	435
1306	CGG AAT AAG CCT ATT ATC TTC TCA ATG GCA AGA TTG GAC CGT GTG	1350
436	Arg Asn Lys Pro Ile Ile Phe Ser Met Ala Arg Leu Asp Arg Val	450
1351	AAG AAC ATG ACA GGT CTA GTT GAG CTA TAT GGA AGG AAT GCC CGT	1395
451	Lys Asn Met Thr Gly Leu Val Glu Leu Tyr Gly Arg Asn Ala Arg	465
1396	CTG AGG GAA CTG GTC AAC CTT GTC GTT GTG GCT GGA GAC CAT GGG	1440
466	Leu Arg Glu Leu Val Asn Leu Val Val Val Ala Gly Asp His Gly	480
1441	AAG GAA TCG AAG GAT CTT GAG GAA CAA GAA GAG CTA AAG AAG ATG	1485
481	Lys Glu Ser Lys Asp Leu Glu Glu Gln Glu Glu Leu Lys Lys Met	495
1486	TAC AGG CTC ATT GAT CAG TAC AAA TTG AAT GGC CAG ATC CGC TGG	1530
496	Tyr Arg Leu Ile Asp Gln Tyr Lys Leu Asn Gly Gln Ile Arg Trp	510
1531	ATC TCT GCC CAG ATG AAC AGG GTT CGC AAT GGT GAG CTA TAC CGC	1575
511	Ile Ser Ala Gln Met Asn Arg Val Arg Asn Gly Glu Leu Tyr Arg	525
1576	TAC ATT GCC GAT ACC GGA GGA GCC TTT GTT CAG CCG GCA TTT TAT	1620
526	Tyr Ile Ala Asp Thr Gly Gly Ala Phe Val Gln Pro Ala Phe Tyr	540
1621	GAA GCA TTT GGG CTC ACA GTT ATC GAA GCC ATG ACG TGT GGC CTG	1665
541	Glu Ala Phe Gly Leu Thr Val Ile Glu Ala Met Thr Cys Gly Leu	555
1666	CCC ACA TTT GCA ACA GCC AAC GGA GGG CCA GCT GAG ATC ATA GTT	1710
556	Pro Thr Phe Ala Thr Ala Asn Gly Gly Pro Ala Glu Ile Ile Val	570
1711	CAT GGC GTT TCC GGC TTC CAC ATT GAT CCT TAC CAG GGT GAC AAA	1755
571	His Gly Val Ser Gly Phe His Ile Asp Pro Tyr Gln Gly Asp Lys	585

1756	GCT GCT GAG CTT CTA GTC AGT TTC TTC GAG AAA TGC AGG GAA GAC	1800
586	Ala Ala Glu Leu Leu Val Ser Phe Phe Glu Lys Cys Arg Glu Asp	600
1801	CCC ACC CAC TGG CAT AAA ATT TCA CAA GGA GGG CTG AAG AGT ATC	1845
601	Pro Thr His Trp His Lys Ile Ser Gln Gly Gly Leu Lys Ser Ile	615
<div style="background-color: yellow; width: 250px; height: 15px; margin: 5px 0;"></div>		
1846	GAG GAG AAG TAC ACC TGG AAG	1866
616	Glu Glu Lys Tyr Thr Trp Lys	

Figure 4. Sequence of partial cDNA of *Borassus flabellifer* *SUS* putative gene. The yellow arrow indicates forward primer, SSFw, and the green arrow indicates reverse primer, SSRv. The red arrow indicates internal primer p1, and the blue arrow indicates internal primer p2. The red box indicates restriction site of *EcoRI* endonuclease restriction enzyme. The brown box indicates putative phosphorylation site (30-RHLSS-34). The blue boxes indicate four putative UDP binding pockets (166-DTGGQ-170; 444-MAR-446; N520; and T546)



Figure 5. The BfSUS putative polypeptide sequence with total length 622 amino acids was generated by translating the sequence of *BfSUS* cDNA, using Geneious 4.6.2.

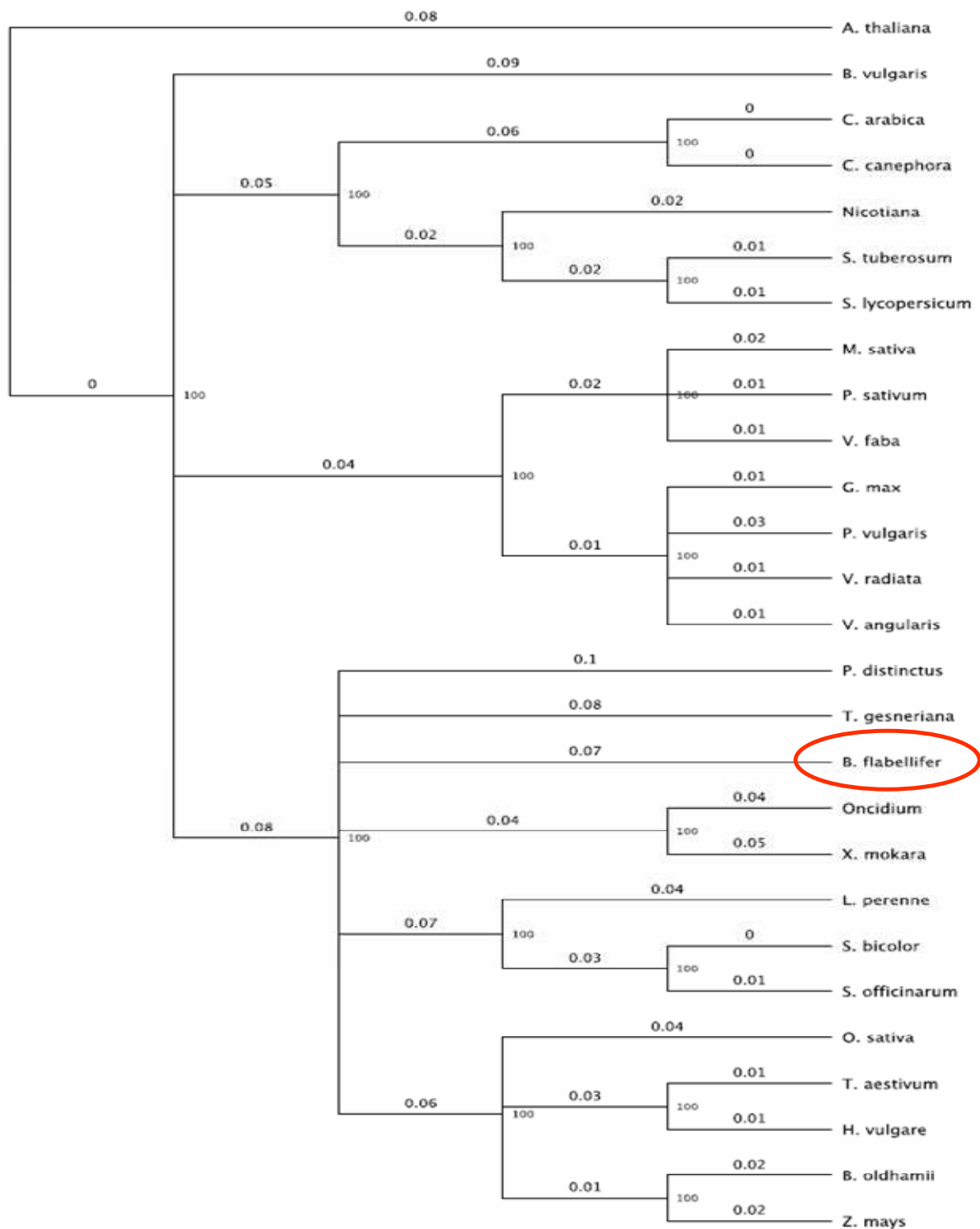


Figure 6. A phylogenetic tree based on sucrose synthase polypeptide sequence of various monocot and dicot species. Similarity of SUS polypeptide among monocot species is distinctively higher than those of dicot species. *Borassus flabellifer* is clustered with the monocot group, although not specifically grouped with particular monocot species. *B. flabellifer* showed the nearest genetic distance with *Tulipa gesneriana*.

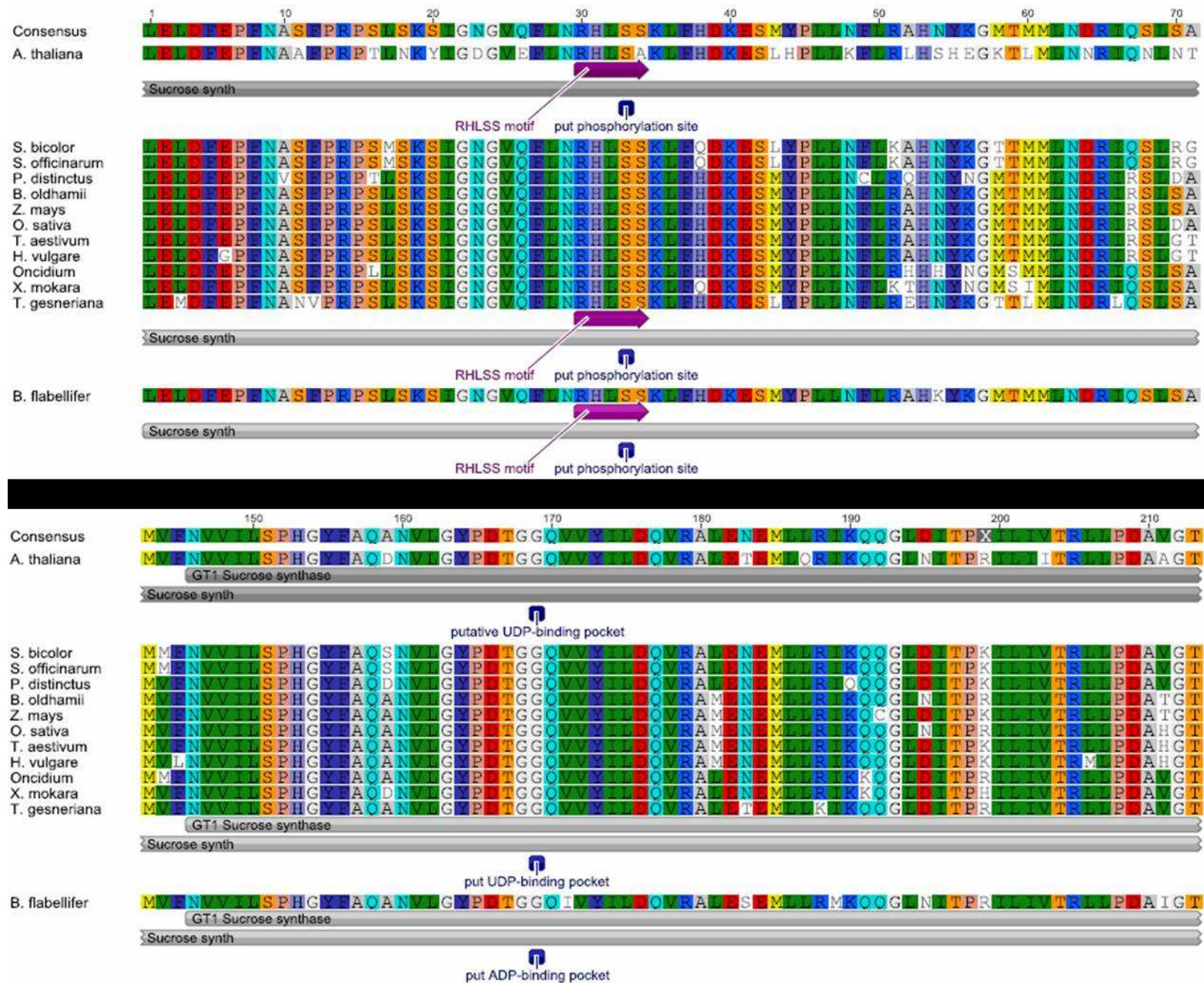


Figure 7. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3)Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

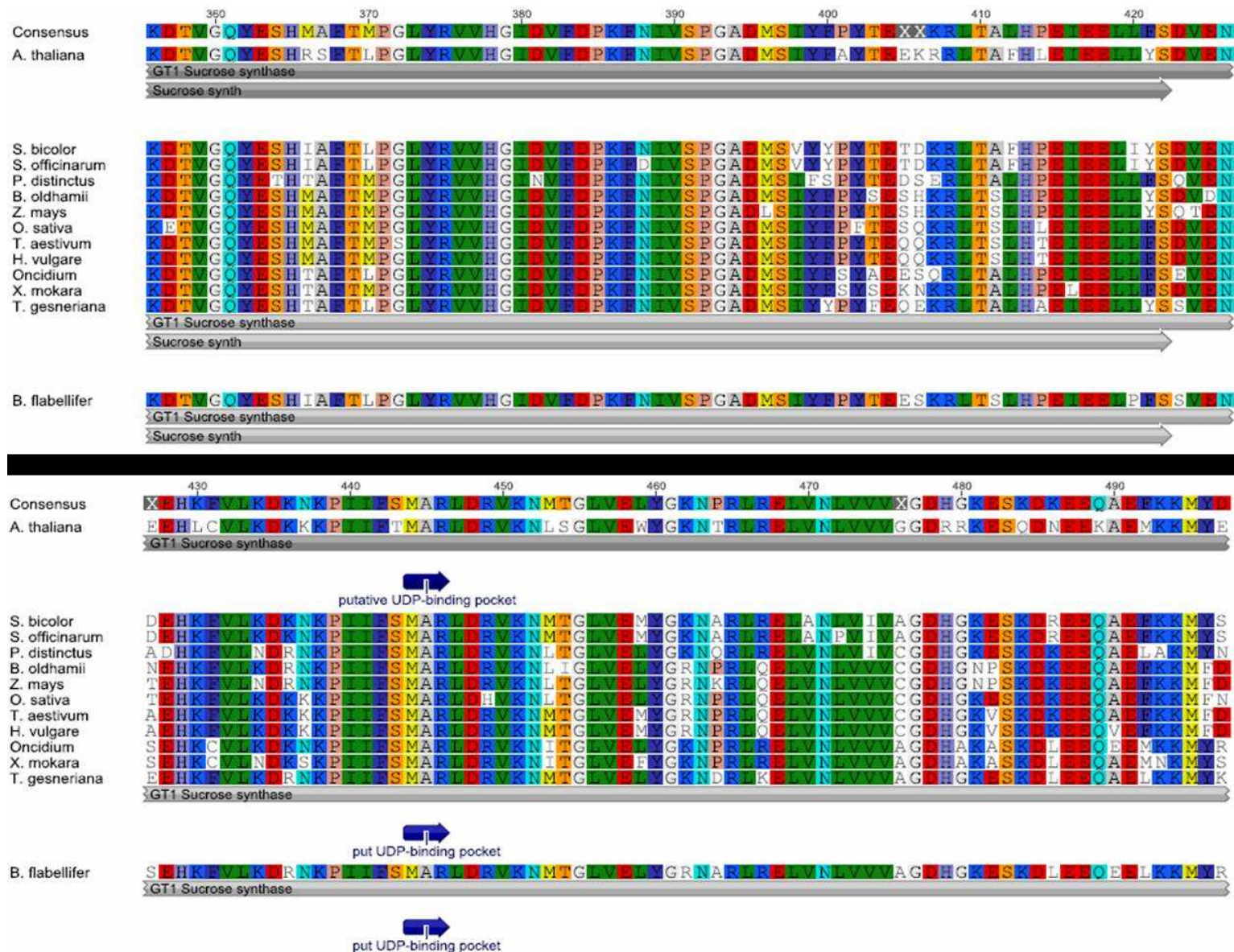


Figure 8. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3) Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

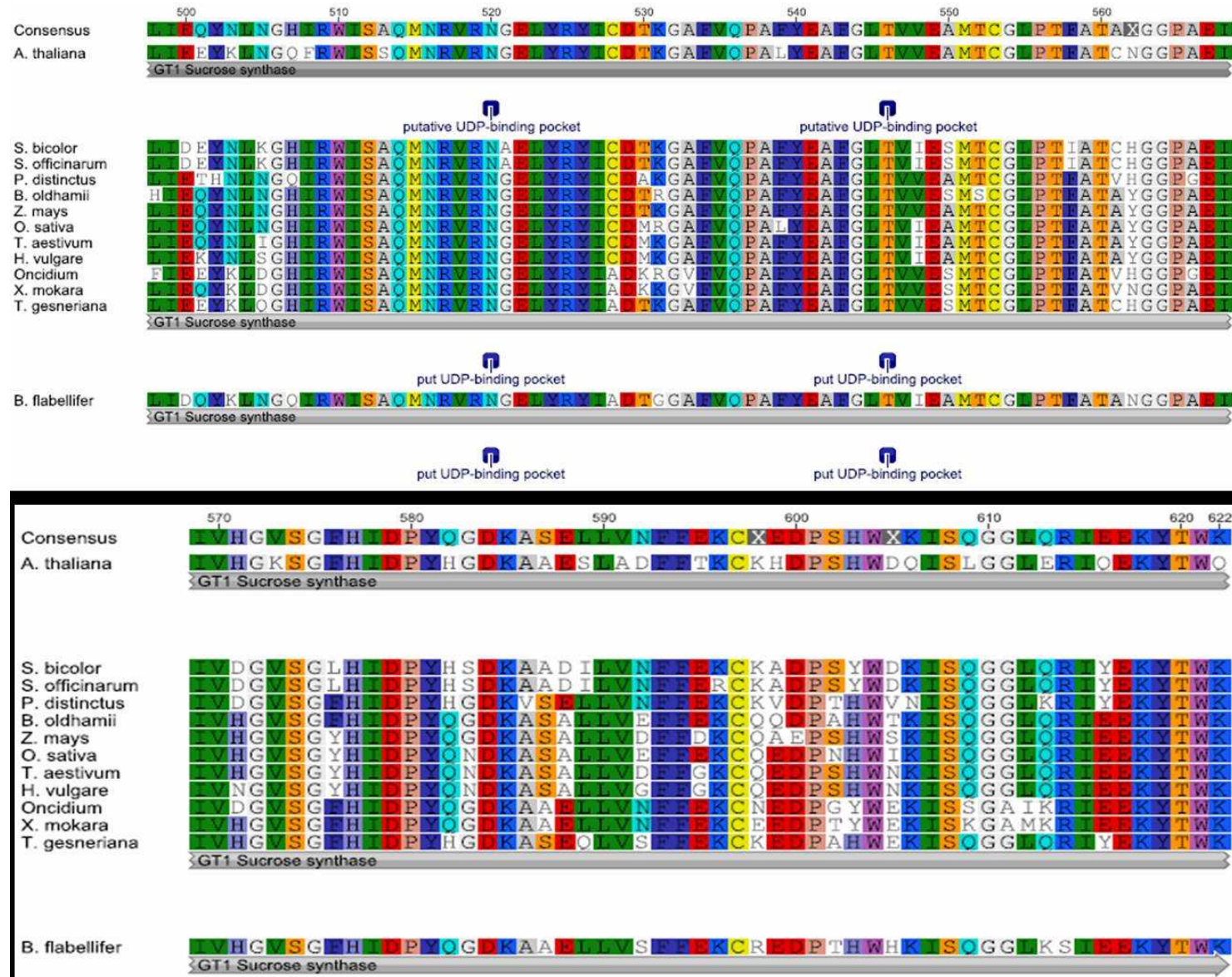


Figure 9. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3) Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

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